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Novel, Rapid, and Inexpensive Cell-Based Quantification of Antimalarial Drug Efficacy

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We report on the development of a new SYBR Green I-based plate assay for analyzing the activities of antimalarial drugs against intraerythrocytic *Plasmodium falciparum*. This assay is considerably faster, less labor-intensive, and less expensive than conventional radiotracer (e.g., [^{3}H]hypoxanthine and [^{3}H]ethanolamine)-based assays or *P. falciparum* lactate dehydrogenase activity-based assays. The assay significantly improves the pace at which antimalarial drug discovery efforts may proceed.

The continued emergence and spread of multidrug-resistant strains of *Plasmodium falciparum* and *P. vivax* are arguably the most pressing problems in the area of infectious diseases today. Also, although the recent deciphering of the *P. falciparum* genome reveals many promising new drug targets, the financial cost of bringing drugs to the clinic is a major obstacle in the development of new antimalarials (6). A faster, less expensive, high-throughput means of screening the activities of drugs against a variety of malarial parasite strains would greatly assist preclinical drug development.

Quantitative assessment of the effects of drugs on parasite growth and development can be achieved by direct (but extremely tedious) microscopic examination of blood smears. An alternative assay is measurement of the effect of drug exposure by determination of the level of incorporation of radiolabeled hypoxanthine. While the latter method can be automated, it requires radioactive materials and is not convenient for detection of parasite stage-specific effects. Another assay measures parasite lactate dehydrogenase activity by methods that do not require radioisotopes. However, this assay requires multiple processing steps and expensive reagents and is not particularly cost-effective for large-scale drug screening efforts.

We have thus endeavored to develop more rapid and convenient cell-based assays for quantifying antimalarial drug activities. We have strived to enhance simplicity and reduce cost. In this paper, we report on the development of one such assay that relies on the fluorophore SYBR Green I.

MATERIALS AND METHODS

Cell culture. Asexual culture is routinely performed. Parasite cultures are initiated from stabilates preserved in liquid nitrogen (the level of parasitemia during storage is ≥10%). Following the initiation of a fresh culture, at least two full life cycles (96 h) are completed before parasites are used for assays. In general, cultures are synchronized in the laboratory, and assays are initiated when the parasites are at the ring stage. However, we find that this assay is equally applicable to asynchronous culture and that similar 50% inhibitory concentrations (IC_{50}s) are calculated from data with asynchronous and synchronous cultures (data not shown). Prior to assay initiation, the level of parasitemia of an aliquot of a stock culture is measured by light microscopy following Giemsa staining or by fluorescence-activated cell sorter analysis after staining with propidium iodide. In general, stock cultures with 5 to 10% parasitemias are used so that they are likely to be in the mid-log growth phase. The level of parasitemia is adjusted prior to transfer to incubation wells. The time that the stock culture is exposed outside a proper gas environment (5% CO_2, 5% O_2, 90% N_2) is minimized (≤15 min).

Overall assay design. Positive controls for each assay contained no inhibitor and different levels of initial parasitemia, and negative controls included chloroquine (CQ) at high concentrations (see Results). Typically, wells are run in triplicate and drugs are tested against both CQ-resistant (CQR) and CQ-susceptible (CQS) strains at various concentrations (see Results). Incubations are carried out for one or two parasite life cycles (48 to 96 h). At the end of the test period, a solution of SYBR Green I (diluted to a 20× concentration in phenol red-free complete medium) is added to each well by use of a volume equal to 10% of the final liquid volume in the well. The resulting solutions are mixed with a low-retention pipette and allowed to stand for 30 min in the incubator. However, accurate detection can be done as soon as 1 min after dye addition. In our hands, lysis of red blood cells with detergents (in particular, harsh ionic detergents, such as sodium dodecyl sulfate) can compromise the signal-to-noise ratio. The data presented in this paper were obtained in the absence of any detergents

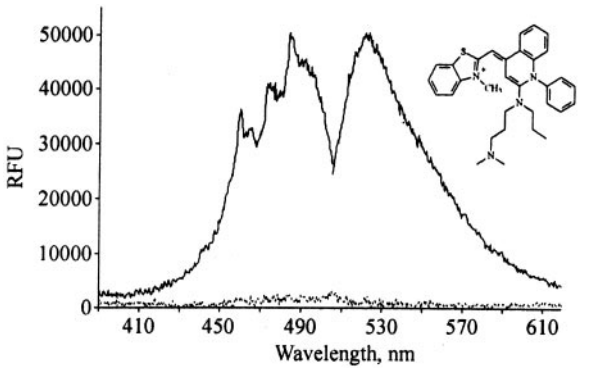

FIG. 1. Fluorescence excitation (390 to 505 nm) and emission (505 to 615 nm) spectra for 7.9 nM SYBR Green I (bottom trace) and 7.9 nM SYBR Green I in the presence of 4.2 nM plasmid DNA base pairs. At this dye:base pair molar ratio (1.9), fluorescence quantum efficiency increases 25- to 30-fold. The increase in SYBR Green I fluorescence quantum yield is linear with molar ratios of 7.0 to 0.5 (assuming approximately equal proportions of GC and AT base pairs; data not shown). RFU, relative fluorescence units. (Inset) Chemical structure of SYBR Green I.

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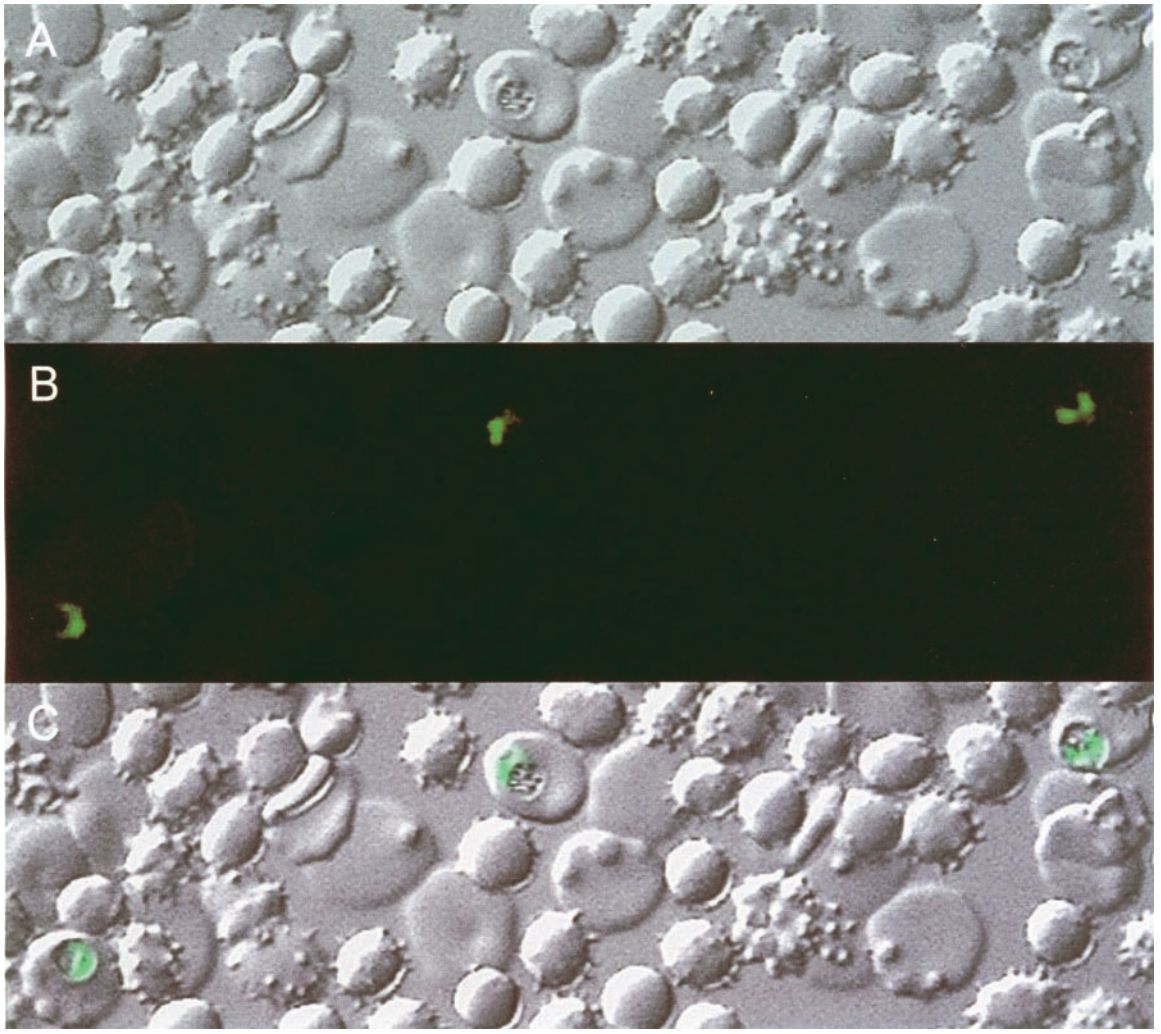

FIG. 2. SYBR Green I staining (in green) of intraerythrocytic malarial parasites attached to thin glass with polylysine and imaged by laser confocal microscopy with an Olympus Fluoview microscope in z-series mode. Excitation is at 488 nm, and emission at 510 to 550 nm is collected with a 505-nm dichroic filter and a combination of appropriate long- and short-band-pass filters. The entire field is bathed in SYBR Green I, but as evidenced by comparison of the differential interference contrast (A), fluorescence (B), and overlay (C), images only parasites are highly fluorescent.

or red blood cell lysis. The SYBR Green I fluorescence emission (530 ± 4.5 nm) from the plates is collected with a Gemini plate reader (Molecular Devices) at an excitation wavelength of 490 nm with 515-nm long-band-pass and 530-nm band-pass emission filters. The Gemini instrument is fully automated, can read 384 well plates, and has a sensitivity of submicromolar concentrations for fluorescence emitters in the visible range. With this reader we find that the use of "black plates" reduces fluorescence cross talk between wells and improves assay reproducibility. To facilitate analysis of the data, we used Softmax Pro software (version 4.1, life sciences edition) and the Excel program.

Assay refinements. We refined the assay to reduce expense and increase both the sensitivity and the reproducibility of the assay. Incubations are typically carried out in 200-μl final volumes in 96-well plates (384-well plates can also be used if automated liquid-handling instrumentation is used). The preferred medium is phenol red-free RPMI to reduce the levels of autofluorescence and background absorbance. We use plates with coated wells to reduce fluorescence cross talk. Measurements depend on the completion of at least one full life cycle (48 to 54 h), which necessitates low initial levels of parasitemia (0.5 to 1.0%) and low levels of hematocrit (1 to 2%). Overall growth inhibition is assessed by comparison of the growth in the treated wells with that in the control wells, to which no drug was added. Several wells on each plate are reserved for use for generation of a control standard curve. To generate the standard curve, a culture with a known level of parasitemia is introduced into these wells immediately prior to dye staining and is stained along with the rest of the plate, so that the absolute SYBR Green I intensities in the test wells can be extrapolated to a defined level of parasitemia. Importantly, such standard curves are linear over a range of 0.2 to 15% parasitemia and overlap for the two strains examined in this study (data not shown). As an additional control, several wells are reserved for initial plating in the absence of drug but at different initial levels of parasitemia (i.e. 0.5, 0.75, and 1.0%). When these controls are stained 48 to 96 h later, they reveal the general health of the culture (e.g., the linearity of growth) within the plate format. If, for example, the control well fluorescence for 1.0% initial parasitemia is not twice that for 0.5% initial parasitemia, the assay is redone.

RESULTS

SYBR Green I (Fig. 1, inset) is a DNA-intercalating dye that prefers G and C base pairs. It can be prepared by heating 2-chloro-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenylquinolinium iodide with *N*-(3-dimethylaminopropyl)-*N*-propylamine in 1,2-dichloroethane for 2 h. The first compound can be rapidly made from 2-chloro-4-methyl-1-phenylquinolinium chloride, which is easily synthe-

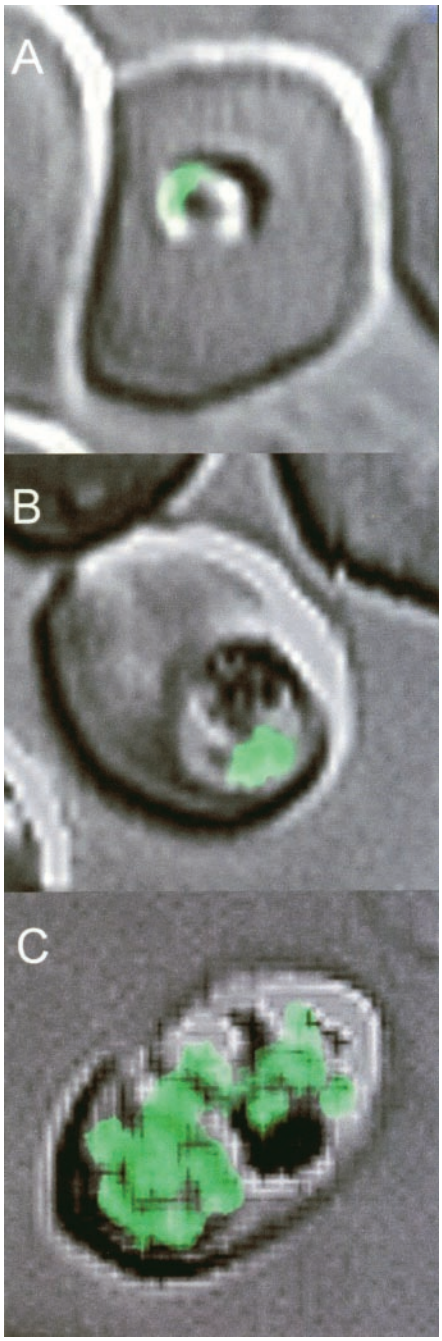

FIG. 3. Stage independence of SYBR Green I staining. By following the procedures described herein, to the best of our ability to ascertain SYBR Green I staining, SYBR Green I stains the ring (A), trophozoite (B), and schizont (C) stages of intraerythrocytic *P. falciparum* equally well. Thus, importantly, this assay is applicable to assessments of the stage-specific effects of drugs with synchronized cultures.

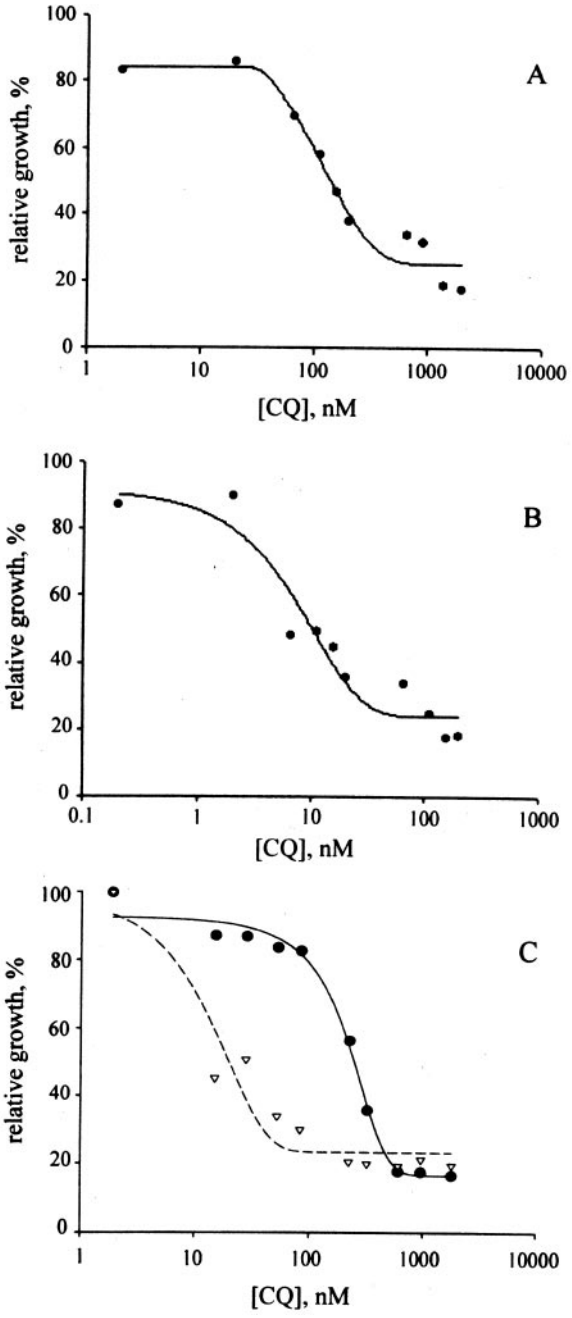

FIG. 4. Representative CQ growth inhibition data for CQR strain Dd2 (A), CQS strain HB3 (B), and CQR strain Dd2 in the absence (closed circles) and the presence (open inverted triangles) of 5 μM VPL (C). The computed IC_{50}s are 125 nM (A), 13 nM (B), and 125 and 21 nM, respectively (C). Thus, importantly, the fold CQ resistance for Dd2 versus that for HB3 obtained by this method agrees very well with many previously published ratios of the IC_{50}s for these strains, as does the fold CQ resistance reversal for strain Dd2 in the presence of VPL.

sized from 1,2-dihydro-4-methyl-1-phenyl-2-quinolone (9). The dye is highly fluorescent when it is intercalated into DNA but is poorly fluorescent when it is not (Fig. 1). Since typical laboratory growth of malarial parasites requires propagation in human red blood cells and since red blood cells do not contain DNA, the use of SYBR Green I and its derivatives thus provides attractive advantages for malarial parasite growth detection assays. Also, the excitation and emission spectra of DNA-intercalated SYBR Green I are broad (Fig. 1), allowing considerable versatility in the choice of detection wavelengths.

Indeed, by laser confocal microscopy with the 488-nm line of

an argon laser, SYBR Green I provides brilliant resolution of infected versus noninfected erythrocytes in asynchronous culture (Fig. 2), even though *P. falciparum* has an AT-rich genome. Importantly, detection is via illumination at convenient visible wavelengths (Fig. 1) and not UV wavelengths, as is the case with many other DNA-staining dyes. Also, very importantly, in Fig. 2 the dye is uniformly exposed to the entire cell culture, but it fluoresces appreciably only when it binds to parasite DNA. Moreover, a high level of fluorescence is seen within seconds after addition of the dye (data not shown). Background staining is minimal and no washing steps are necessary, making use of this dye in a 96-well plate format ideal. That is, in theory, parasites may be grown with or without drug in a traditional red blood cell culture environment, stained by one simple addition of SYBR Green I, incubated for 5 min, and then rapidly read on an appropriate fluorescence-activated plate reader. Initially, results such as those shown in Fig. 2 were combined with those of fluorescence-activated cell sorter analysis to give a robust, quantitative measure of parasite growth and development. We validated this procedure by direct comparison of the results obtained by the procedure with those obtained by light microscopy and have used it extensively for routine measurement of inhibition of merozoite invasion of erythrocytes.

Figure 3 shows that staining with SYBR Green I is similarly intense regardless of the stage of intraerythrocytic parasite development. The intensity per parasite is similar for both rings (Fig. 3A) and trophozoites (Fig. 3B), and multiple SYBR Green I spots for the multiple nuclei present at the schizont stage are readily visible (Fig. 3C, in which 12 new nuclei can be discerned). Since each malarial parasite typically produces 8 to 24 progeny upon completion of intraerythrocytic growth, there should be significant amplification of the net SYBR Green I intensity over time as a culture expands.

Figure 4A to C shows the results of typical growth inhibition assays with CQ for strains HB3 (CQS) and Dd2 (CQR). Data are fit to sigmoidal curves, and the computed IC_{50}s (125 and 13 nM for Dd2 [Fig. 4A] and HB3 [Fig. 4B], respectively) are near those computed by other assays in other laboratories (7). More importantly, the ratio of these IC_{50}s (that is, the fold resistance to CQ for Dd2 versus that for HB3) are nearly identical to the ratios calculated by the use of other assays (e.g., see reference 7 and the references therein). We do not expect exact agreement of the IC_{50}s determined by the different methods, since the different reporters in various assays can have stage- and time-dependent effects. In Fig. 4C we show CQ growth inhibition data for Dd2 with and without verapamil (VPL), which is a well-known chloroquine resistance reversal agent. Importantly, the level of resistance reversal in this assay (approximately sixfold) compares very well with the results from other laboratories in which more traditional assays were used.

DISCUSSION

SYBR Green I and related compounds have been used to either monitor the appearance of DNA in biochemical matrices or the DNA concentration versus time. PCR-based assays for gene detection (1, 5, 8) and detection of DNA repair (3), gel mobility assays (4), and bacterial detection assays (2) have all been developed. Because red blood cells do not harbor DNA and because most antimalarial drugs target the intraerythrocytic stage of parasite development, assays based on similar principles should be helpful for high-throughput screening for new antimalarial therapies.

By the new assay that we have described, staining, quantification of growth inhibition, and calculation of the IC_{50} can be completed within 15 min. The activities of about 40 compounds can be preliminarily screened on a single 96-well plate. With automated pipetting, this expands to 200 compounds/384-well plate. By including the time for cell culture, one person in a typical laboratory can test 20 plates per week. This allows significantly accelerated and less expensive assessments of candidate drugs or drug combinations. After preliminary screening, for example, the activities of 8 to 10 drug pairs can then be rapidly assessed against two different strains (e.g., CQS and CQR strains) per plate. The assay is exceedingly fast because only one reagent is added to the plate after parasite growth and no washing or filtering steps are required. Comparative IC_{50}s are used in preliminary screens of antiparasitic compounds; however, an in vitro therapeutic index is required to describe the selective toxicity for the parasite. Accordingly, we note that the assay described here can also be easily applied to mammalian cell culture using similar principles.

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